Ecotoxicology and Environmental Safety 169 (2019) 950–959

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Responses of *Raphidocelis subcapitata* exposed to Cd and Pb: Mechanisms of toxicity assessed by multiple endpoints

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ARTICLE INFO

Keywords:
Chlorophyceae
Biomarkers
Photosynthesis
PAM fluorometry
ROS
Lipid classes

ABSTRACT

Microalgae have been widely used in ecotoxicological studies in order to evaluate the impacts of heavy metals in aquatic ecosystems. However, there are few studies that analyze the effects of metals in an integrative way on photosynthetic apparatus of freshwater microalgae in the generation of reactive oxygen species (ROS) and biochemical composition. Therefore, this study aimed to assess cadmium (Cd) and lead (Pb) toxicity using synchronously physiological and biochemical endpoints, specially detecting lipidic classes for the very first time during Cd and Pb-exposure to *Raphidocelis subcapitata*. Here we show that analyzing the algae growth, the IC50–72 h for Cd was 0.04 μM and for Pb was 0.78 μM. In general, the Cd affected the biochemical parameters more, leading to an increase in total lipid content (7.2-fold), total carbohydrates (3.5-fold) and ROS production (3.7-fold). The higher production of lipids and carbohydrates during Cd-exposure probably acted as a defense mechanism, helping to reduce the extent of damage caused by the metal in the photosynthetic apparatus. For Pb, the physiological parameters were more sensitive, which resulted in changes of chlorophyll *a* synthesis and a reduction of both efficiency of oxygen-evolving complex and quantum yields. Besides that, we observed changes in the lipid class composition during Cd and Pb-exposure, suggesting these analyses as great biomarkers to assess metal toxicity mechanisms in ecological risk assessments. Thereby, here we demonstrate the importance of using multiple endpoints in ecotoxicological studies in order to obtain a better understanding of the mechanisms of metal toxicity to *R. subcapitata*.

1. Introduction

Metal compounds are widely used in industries and agricultural activities, and therefore metal levels in ecosystems have substantially increased worldwide over the last century. Aquatic environments are particularly susceptible to accumulating different metals, and since microorganisms cannot degrade metallic compounds, these contaminants may exert toxic effects to various organisms by waterborne exposure or *via* transfer through food webs (Chen et al., 2000; Hook and Fisher, 2001), even threatening human health. Metal toxicity has consequences such as an increase in lethality, inhibition of growth rates, physiological alterations and production of reactive oxygen species (ROS) in phytoplankton for the communities at the base of the aquatic food webs (Carfagna et al., 2013; Qian et al., 2009; Zhou et al., 2006) and in zooplankton (Kim et al., 2014; Lari et al., 2017; Martins et al., 2017; Zeman et al., 2008), which may have an impact on the entire food web. In addition, heavy metals can be bioaccumulated in higher trophic levels, as observed by Chen et al. (2000) and Rubio-Franchini et al. (2016).

Non-essential metals, such as cadmium (Cd) and lead (Pb) can enter the aquatic ecosystems from various sources. Lead is a very toxic metal that can enter in natural waters through different sources, such as industrial fabrication, paints, smelting of metallic ores, explosives and mainly leaded gasoline (Scheidegger et al., 2011; Sharma and Dubey, 2005). Lead concentrations may greatly vary in different aquatic environments, such as 6.27×10^{-10}–4.18×10^{-7} M Pb for the Pardo

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https://doi.org/10.1016/j.ecoenv.2018.11.087
Received 4 July 2018; Received in revised form 17 October 2018; Accepted 19 November 2018

River, Brazil (Alves et al., 2014) and 9.65×10^{-11}–1.05×10^{-8} M Pb for the Tarim River Basin, China (Xiao et al., 2014). However, anthropogenic actions can increase metal levels in water, *e.g.* the recent collapse of *Fundão*, an iron ore tailing dam in Brazil belonging to a mining industry (Samarco Business Corporation), greatly increased levels of metals, including Pb, in the Doce River estuary (Brazil, Marinha do Brazil, 2016, 2017). On the other hand, cadmium (Cd) is a widely distributed metal mainly due to anthropogenic actions, which correspond to 90% of Cd found in surface water. Cadmium can be found at low concentrations in aquatic environments, *e.g.* < 8.89×10^{-11}–4.44×10^{-10} M Cd in the Vacacaí River, Brazil (Kochhann et al., 2013). However, natural activities and remobilization of existing mineral sources (US EPA - United States Environmental Protection Agency, 2016; WHO - World Health Organization, 2010) could increase Cd concentrations to levels exceeding 9×10^{-6} M Cd (ATSDR, 2012), and is highly toxic to most organisms, including humans (Silverberg, 1976). Thus, Cd and Pb are possible threats to aquatic organisms (Töpperwien et al., 2007).

Microalgae are of great importance in aquatic environments since, as primary producers, they provide oxygen and organic substances to other life forms, and are essential for maintaining the ecological balance of these ecosystems. It is known that Cd and Pb exposure can induce adverse effects on microalgae physiological and biochemical processes, *e.g.* chlorolysis induction, growth inhibition, damage of the photosynthetic apparatus, cell death and changes in biovolume, pigment levels and biochemical contents (Carfagna et al., 2013; Debelius et al., 2009; Miao et al., 2005). Besides that, it has been shown that Pb can enter into algal cells through calcium or magnesium channels (Scheidegger et al., 2011) and may accumulate in them (Debelius et al., 2009).

In addition, metals can also increase ROS in algal cells, thereby generating changes in proteins, DNA and lipids, that can cause cell death. Chloroplasts are the main source of ROS, which include free radicals, such as hydroxyl radical ($OH^{\bullet}$), phenoxy radicals ($RO^{\bullet}$), peroxy radicals ($ROO^{\bullet}$), superoxide radical anion ($O2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Qian et al., 2009). In order to avoid the damage caused by oxidative stress, algae have enzymatic and non-enzymatic defense systems that can minimize the intracellular ROS concentration (Carfagna et al., 2013).

Microalgae biochemical composition can change under stress conditions, *e.g.* limitation or excess of nutrients (Rocha et al., 2018; Ross et al., 2018), herbicides contamination (Prado et al., 2009) and metal stress (Miao and Wu, 2006). In microalgae, the lipids produced can act as storage lipids (non-polar), mainly in the form of triglycerides (TAG), and structural lipids (polar) (Sharma et al., 2012a). The stress caused by the toxicity of some compounds, especially metals, may lead the algae to alter their metabolic pathway of lipid production, modifying the amount and composition of lipid classes (Rocha et al., 2016). Therefore, a higher lipid production can be an indicator of metal stress, especially if there is accumulation of neutral lipids in the form of TAG (Chia et al., 2013). The inclusion of biochemical composition analyses could improve ecotoxicological studies, providing important information on the physiological and biochemical responses of algae, in order to assess mechanisms and sites of action of contaminants in the algal cells.

Furthermore, recently new tools such as the technique of pulse-amplitude-modulated (PAM) fluorescence have been used to evaluate the photosynthetic parameters of algae and plants, and to understand the effects and extent of the toxicity of metals in photosynthetic apparatus (Echeveste et al., 2017; Herlory et al., 2013; Silva et al., 2018). Moreover, with PAM fluorescence it is possible to infer how some toxic compounds interfere with the photosynthetic processes and the sites of action of these compounds through maximum quantum yield measurements (ϕ_M) and the effective quantum yield (ϕ'_M) of photosystem II (PSII) (Miao et al., 2005).

Despite available information in the literature about Cd and Pb toxicity to microalgae, only a few studies analyze biochemical and physiological endpoints synchronously, especially including lipid class measurements. The search for more sensitive endpoints in ecotoxicological studies, especially in microalgae, which being at the base of the food webs, are the first targets affected by metals, and the understanding of the level (biochemical, molecular or physiological) in which these organisms begin to be affected, may help to formulate more effective environmental protection guidelines.

Thereby, we aim to investigate the toxicity mechanisms of Cd and Pb to the green algae *Raphidocelis subcapitata* (= *Selenastrum capricornutum*) simultaneously using biochemical (total carbohydrate and lipid contents, lipid composition and ROS generation) and physiological endpoints (growth inhibition, chlorophyll *a* content and PAM-parameters). This Chlorophyceae was chosen as a test organism because of its greater sensitivity compared to other algae species (De Schamphelaere et al., 2014). In this study, we intend to demonstrate that lipidic classes are good biomarkers to assess Cd and Pb toxicity mechanisms and, as far as we know, this is the first study reporting changes in lipidic composition of *R. subcapitata* exposed to these metals.

2. Material and methods

2.1. Algae cultures and toxicity tests

The Chlorophyceae *R. subcapitata* was cultured in half-strength Chu 10 (Nalewajko and O'Mahony, 1989) previously autoclaved (121 °C, 1 atm above standard pressure, 20 min). Exponential growth cultures were inoculated in a concentration of 10^5 cells mL^{-1} in 500 mL polycarbonate erlenmeyers containing 250 mL of test solutions. All polycarbonate flasks were immersed in 10% hydrochloridric acid for 7 days and rinsed thoroughly in deionized water before use, to avoid metal contamination.

The treatments consisted of a control group (no metal addition) and nominal concentrations of Cd (0.02; 0.04; 0.08 and 0.17 μM) and Pb (1.20; 2.41; 4.82 and 12.06 μM). Stock solutions of Cd (1000 mg Cd L^{-1}) and Pb (1000 mg Pb L^{-1}) were obtained from AAS standard solutions of $CdCl_2$ and $Pb(NO_3)_2$ (Merck, Germany). The toxicity test, with triplicates, was maintained in a controlled room at 25 ± 2 °C, in a 12:12 h light: dark photoperiod with ≅ 3900 lx of fluorescent light intensity for 72 h, when sampling for biochemical composition, ϕ'_M and quenching parameters were taken. Cell densities were monitored every 24 h. During the test, the erlenmeyers were manually shaken 3 times a day.

2.2. Cell density and ROS measurements

The relative ROS assessment followed procedures proposed by Hong et al. (2009), with modifications described as follows: during test daily measurements, 5 μL of DCFH-DA (2′,7′-Dichlorofluorescein diacetate, CAS number 2044–85-1, Sigma Aldrich) diluted in dimethylsulfoxide (10^4 μM) were added to 495 μL of each sample to obtain a final concentration of 10 μM. Samples were incubated for 1 h in the dark at room temperature and then analyzed in a FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW blue-argon ion laser (488 nm excitation). For relative ROS determination, we used parameters FL3-H and FL1-H (green fluorescence). For cell density, we used parameters SSC-H (lateral dispersion) *versus* the fluorescence intensity of FL3-H channel (red fluorescence), according to procedures established by Sarmento et al. (2008). For both cell density and ROS, immediately before cytometer analysis we added 10 μL of 6 μm fluorescent beads (Fluoresbrite carboxylate microspheres; Polysciences, Warrington, Pennsylvania, USA) to the samples as an internal standard. The raw data was analyzed in FlowJo software, version V10.0 (Treestar.com, USA).

The relative ROS (%) were calculated by two equations. Firstly, we calculated the relative FL1-H (Eq. (1)):

$$FL1 - H_{relative} = \log(FL1 - H_{samples}) / \log(FL1 - H_{beads}) \quad (1)$$

After that, relative ROS (%) was calculated according to (Hong et al., 2009) equation (Eq. (2)):

$$ROS_{relative}(\%) = (FL1 - H_{relative[treatments]} / FL1 - H_{relative[control\ group]}) \times 100 \quad (2)$$

2.3. Biochemical analysis

Biochemical analyses were carried out after 72 h-exposure. Total carbohydrates were quantified according to the phenol-sulphuric reaction method proposed by Liu et al. (1973). Samples were analyzed using a spectrophotometer (HACH DR 5000; HACH Company, Loveland, CO, USA) at a wavelength of 485 nm. After that, carbohydrate values were calculated using a calibration curve (Fig. S1, Supplementary material), with 7 concentrations (5, 10, 20, 40, 100, 150 and 200 μg mL^{-1}) of dextrose anhydrous (Mallinckrodt Chemicals, USA) as a standard.

Total lipids were extracted and quantified using the modified method of Folch et al. (1957), described by Parrish (1999). Algal samples (100 mL) were filtered in pre-baked (400 °C, 8 h) glass fiber filters (Macherey-Nagel, Germany), and stored (< 5 days) at −20 °C until extraction. After extraction in chloroform: methanol: chloroform extracted water (2:1:1), with 5 min of sonication (Unique Group, Brazil) and 2 min of centrifugation at 3000 rpm (Eppendorf 5702 R, Germany), samples were concentrated using a rotary vapor (Buchi Rotavapor R, Buchi Labortechnik AG, Switzerland). The identification and quantification of the lipid classes were performed by thin layer chromatography with flame ionization detection (TLC / FID), in an Iatroscan MK6 (Mitsubishi, Kagaku Iatron Inc., Tokyo, Japan), using patterns (Sigma-Aldrich, USA) detected as lipid peaks (Fig. S2, supplementary material). All glass (baked 400 °C, 8 h) and Teflon flasks were washed 3 times with methanol and 3 times with chloroform before use, to avoid lipid contamination to the samples.

2.4. Determination of chlorophyll a content

The chlorophyll *a* (Chl *a*) content was determined according to Shoaf and Lium (1976), with extraction based on the reaction with dimethylsulfoxide (DMSO). From each triplicate, 10 mL of the sample was filtered onto cellulose ester membranes (0.45 μm pore size). The absorbance of the samples, after a period of 45 min in the dark with periodic shaking, was read at 664 nm and 630 nm through a HACH DR500 spectrophotometer (HACH Company, Loveland, CO, USA). From the absorbance data, the Chl *a* content was calculated based on the equation described in Jeffrey and Humphrey (1975).

2.5. Photosynthetic activity and fluorescence parameters

Chlorophyll *a* fluorescence was measured using an amplitude-modulated fluorometer (Phytoplankton Fluorometer Analyzer, Phyto-PAM, Heinz Walz GmbH, Germany), equipped with an optical drive ED-101US/MP. The analyzed parameters are described in Table 1.

The ϕ_M of PSII was measured daily, while the ϕ'_M was determined after 72 h of exposure. The minimum (or initial) fluorescence of the dark-adapted algal cells (F_0) was measured using modulated light of low light intensity (< 0.3 μmol photons m^{-2} s^{-1}) (Genty et al., 1989) in order to avoid the reduction of the primary electron acceptor of the PSII (Q_A). The maximum fluorescence of dark algae (F_M) was quantified by the complete reduction of all the Q_A, using a short saturating pulse of light (2600 μmol photons m^{-2} s^{-1}). Through the difference between F_0 and F_M, the variable fluorescence (F_V) was determined and the ϕ_M of PSII (F_V/F_M) was calculated (Schreiber, 1986; Schreiber and Bilger, 1993). The efficiency of the oxygen evolving complex (OEC) of PSII (F_0/F_V) was also calculated from the fluorescence emission of dark-acclimated algal cells (Kriedemann et al., 1985).

Parameters from the photosynthetic performance of algal cells were obtained from fluorescence induction kinetics (Herlory et al., 2013). Dark-acclimated samples were illuminated by an actinic light at an intensity of 64 μmol photons m^{-2} s^{-1} for 15 min, followed by the application of a saturation pulse every 20 s. From the values of maximum fluorescence in the light (F_M') and the initial fluorescence in light (F_S), the parameters of variable fluorescence in the state adapted to the light ($F_V' = F_M' - F_S$), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN e NPQ), and the ϕ'_M of PSII were calculated. The values of F_0, F_M, ϕ_M, F_M' and ϕ'_M were provided by the equipment.

The qP estimates the ability of photochemistry to compete for imprisoned quantum energy. The qN reflects the dissipation of light energy unrelated to photochemistry. The NPQ, another non-photochemical quenching measurement parameter, represents the relative increase in the sum of the activation rates of non-photochemical processes (such as fluorescence, heat dissipation and excitation energy transfer from PSII to photosystem I) (Cosgrove and Borowitzka, 2010; Juneau et al., 2002; Miao et al., 2005).

2.6. Metal determination

Firstly, stock solutions of Cd (10 mg L^{-1}) and Pb (1000 mg L^{-1}) used in toxicity tests were acidified with 1% (v/v) ultrapure HNO_3. The samples were measured by flame atomic absorption spectrometry (PerkinElmer PinAAcle 900 T, USA), burner 50 mm. Cadmium and Pb were quantified using 228.80 and 283.31 nm absorption lines, 0.7 nm slit width, with flame stoichiometry of 10.0 and 2.5 L min^{-1} of synthetic air and C_2H_2, respectively. We used the nominal concentrations of Cd and Pb in this study, since they did not differ more than 10% from the measured concentrations (Tables S1 and S2, Supplementary material).

In addition, we used MINEQL + (version 4.6) chemical equilibrium program to estimate Cd and Pb free ions in the test solutions (Tables S3 and S4, Supplementary material). The model considers the total heavy metals and ligand concentrations present in the culture media, as well as the affinity constants between the different ligands, heavy metals and solubility product constants (Machado et al., 2015).

2.7. Statistical analyses

Data were tested for normality using the Shapiro-Wilk test and equal variance with the Levene median test. Normal distributed data were

Table 1
Fluorescence parameters calculated from PAM fluorometry measurements (modified from Herlory et al., 2013).

Parameter	Definition	Equation	Reference
ϕ_M	Maximum quantum efficiency of photosystem II (PSII)	$(F_m-F_0)/F_m$	Schreiber (2004)
F_o/F_V	Efficiency of the oxygen-evolving complex	$F_0/(F_m-F_0)$	Kriedemann et al. (1985)
ϕ'_M	Effective quantum efficiency of PSII	$(F_m'-F_S)/F_m'$	Baker (2008); Cosgrove and Borowitzka (2010); Genty et al. (1989)
qP	Photochemical quenching	$(F_m'-F_S)/(F_m'-F_0')$	Bilger and Schreiber (1986); Juneau and Popovic (1999)
qN	Non-photochemical quenching	$1-(F_m'-F_0')/(F_m-F_0)$	Bilger and Schreiber (1986); Juneau and Popovic (1999)
NPQ	Non-photochemical quenching	$(F_m-F_m')/F_m'$	Bilger and Björkman (1990)

analyzed using One-Way ANOVA and Dunnett's and Tukey post-hoc tests. The data with non-normal distribution or unequal variance were analyzed using the Kruskal-Wallis test and non-parametric Dunnett's and Tukey post-hoc tests. Statistical significances were considered for $p < 0.05$ level. A correlation matrix-based principal component analysis (PCA) was used to determine the correlation between analyzed parameters (PAST 3.14) (Fig. S3, Supplementary material). Inhibitory concentration (IC) values were calculated using a specific growth rate at 72 h (Eq. (3) – US EPA - United States Environmental Protection Agency, 2012) with a linear interpolation method using ICPIN 2.0 software (USEPA, Duluth, MN, USA), with 80 resamples.

$$\mu = [\ln(b_f) - \ln(b_i)]/t \quad (3)$$

where: μ = specific growth rate (day^{-1}); b_f = observed biomass (cell density) at the end of the experiment; b_i = observed biomass (cell density) at the beginning of the experiment; t = time of exposure (in days).

3. Results

3.1. Free ion determination

According to the MINEQL$^+$ speciation analysis, in Cd-treatments, 98.99% of the metal added was dissociated as Cd^{2+}. At Pb treatments, only 69.83% of metal added was dissociated as Pb^{2+}, while ≅19.65% and 6.22% were complexed into $PbOH^+$ and $PbCO_{3(aq)}$ forms, respectively.

3.2. Cell density and growth inhibition

The IC10–72 h for Cd and Pb were 0.009 and 0.150 μM, respectively. Cadmium and Pb significantly inhibited the growth of *R. subcapitata* (Fig. 1), thereby all treatments differed significantly ($p < 0.05$) from the control group at 72 h of exposure. Besides that, our results showed that Cd was about 19-fold more toxic to the algae than Pb, with IC50–72 h values of 0.04 μM for Cd and 0.78 μM for Pb (Table 2). At 0.04, 0.08 and 0.17 μM of Cd, algae showed a sudden increase of growth rates from 24 h to 48 and 72 h. However, in Pb treatments there were no oscillation in growth curves.

3.3. Photosynthetic parameters

In general, we observed F_0/F_V (efficiency of the OEC) increased values over time at the highest Cd and Pb concentrations (Fig. 2a and b). In algae exposed to Pb, F_0/F_Vincreased 62.2%, while in Cd-exposure a less significant increase of 43.6% was observed for the same parameter. We also observed a reduction in the Φ_M values, which indicates that only 56% (at 0.17 μM of Cd) and 54% (at 12.06 μM of Pb) of the light absorbed by *R. subcapitata* could be used in photosynthesis. Moreover, Pb led to changes in Φ_M at all treatments. Cadmium decreased the effective Φ'_M values at the two highest concentrations, as shown in Fig. 2c–f.

There was no significant change ($p > 0.05$) in qP values for both metals (Fig. 2g and h). In addition, significant effects in qN at Pb-exposure and in NPQ at Cd-exposure were not observed. The qN only had a significant increase ($p < 0.05$) in *R. subcapitata* exposed to the highest Cd concentration (0.17 μM); while NPQ was significantly lower ($p < 0.05$) at concentrations of 1.20, 2.41 and 12.06 μM of Pb.

Table 2
Inhibitory concentrations (IC) values for *Raphidocelis subcapitata* after 72 h of exposure to cadmium (Cd) and lead (Pb). IC values were calculated through ICPIN 2.0 software (USEPA, Duluth, MN, USA), with 80 resamples. Results correspond to median and 95% confidence intervals. Results correspond to median and 95% confidence intervals.

At 72 h of exposure	Concentrations (μM)	
	Cd	Pb
IC10	0.009 (0.002–0.030)	0.15 (0.14–0.17)
IC25	0.020 (0.006–0.042)	0.39 (0.35–0.45)
IC50	0.04 (0.03–0.06)	0.78 (0.72–0.88)

3.4. Chlorophyll a content and ROS measurements

We observed an increase of Chl *a* concentrations in Cd and Pb exposures (Fig. 3a and b), with a significant ($p < 0.05$) increase at the highest concentrations of Cd (0.17 μM) and Pb (12.06 μM). Cadmium induced the formation of ROS (*e.g.* Fig. S4, supplementary material) at 0.02 and 0.04 μM after 24 h of exposure; while in 48 and 72 h-exposure, all Cd treatments differed ($p < 0.05$) from the control group (Fig. 3c and d). Curiously, after 72 h-exposure, significantly ($p < 0.05$) higher ROS formation was observed at the lowest Cd concentrations (0.02, 0.04 and 0.08 μM) than at 0.17 μM. Lead induced ROS increased ($p < 0.05$) only in 24 and 48 h of exposure, although after 72 h-exposure, Pb treatments did not differ from the control group.

3.5. Biochemical composition

Cadmium and Pb significantly affected ($p < 0.05$) the total lipids of *R. subcapitata* (Table 3) at the two highest Cd concentrations and in all Pb treatments. It was observed that the greatest increase in lipid levels occurred in Cd-exposure, especially at 0.17 μM, corroborating Chia

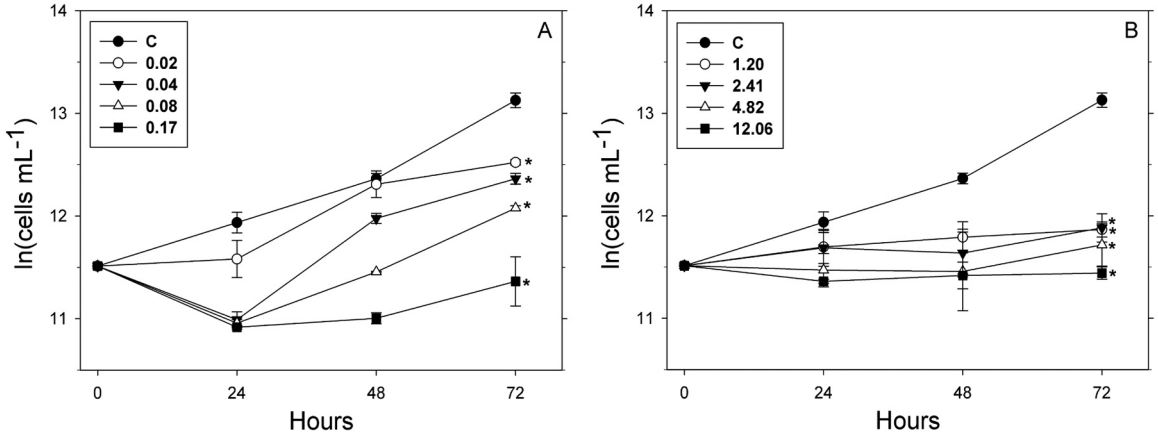

Fig. 1. Cell density of *Raphidocelis subcapitata* (ln(cells mL^{-1})) exposed to cadmium (A) and lead (B) during 72 h. Concentrations are expressed in μM, where: C = control group and asterisks represent a significant difference ($p < 0.05$) of treatments compared to the control group.

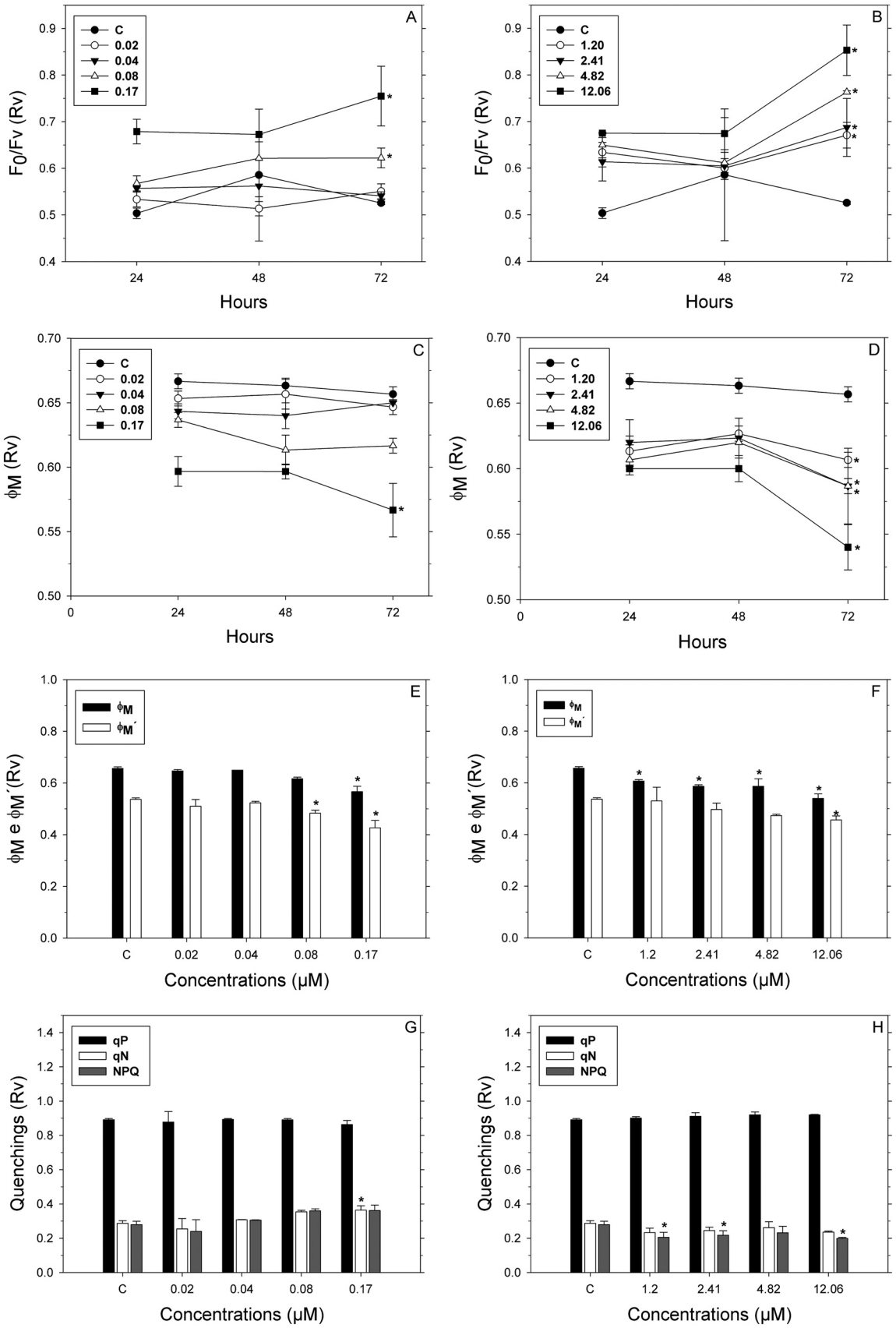

Fig. 2. Photosynthetic (ϕ_M, F_0/F_V and ϕ'_M) and quenching parameters (qP, qN and NPQ) of *Raphidocelis subcapitata* exposed to cadmium (A, C, E, G) and lead (B, D, F, H). Parameters presented in A-D were measured daily, while E-H data were obtained at 72 h. Concentrations are expressed in μM, where: C = control group and asterisks represent a significant difference ($p < 0.05$) of treatments compared to the control group for each data set.

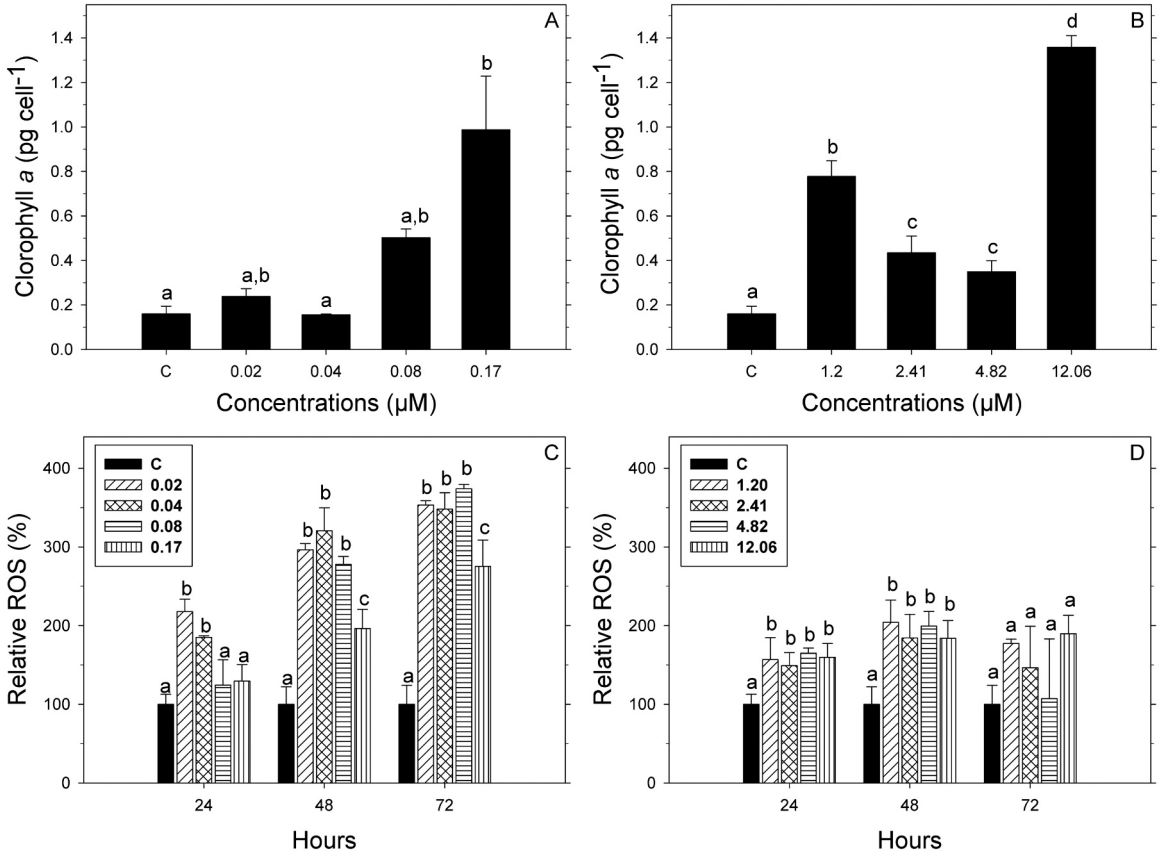

Fig. 3. *Raphidocelis subcapitata* exposed to cadmium (A, C) and lead (B, D), concentrations are expressed in μM. Letters A and B refer to Chlorophyll *a* content (pg cell^{-1}); C and D refer to reactive oxygen species (ROS). Measurements were made after 72 h of exposure. Different letters represent a significant difference ($p < 0.05$) in A and B, and it represents a significant difference ($p < 0.05$) for each data set in C and D.

et al. (2015), who also reported higher lipid production of *Chlorella vulgaris* exposed to Cd. Moreover, Cd and Pb also affected lipid composition, both altering ($p < 0.05$) the levels of HC (aliphatic hydrocarbons), TAG, FFA (free fatty acids), ALC (aliphatic alcohol) and PL (phospholipids), although ST (free sterol) and AMPL (acetone-mobile polar lipids) were altered only by Cd exposure. At 0.17 μM of Cd, the highest lipid classes increase were FFA (18.1- fold), followed by ST (12.3- fold) and TAG (12.4- fold). At 12.06 μM of Pb, the lipid classes that most increased were FFA (9.8-fold), ALC (6-fold) and HC (5.3-fold). In addition, total carbohydrates were an efficient endpoint to assess Cd toxicity, since a 3.5-fold increase of algae carbohydrates was observed at 0.17 μM. Nevertheless, there was no significant change in the carbohydrate levels during Pb-exposure.

4. Discussion

The effects of Cd and Pb on *R. subcapitata* included growth inhibition, ROS generation, damage to the photosynthetic apparatus and alteration of biochemical composition. We identified the toxicity mechanisms of both metals through multiple endpoint analysis, in which lipid classes were highly important to elucidate how the algae had changed its metabolic pathways in response to each metal.

4.1. Cell density and growth inhibition

In our study, Cd and Pb completely inhibited algal growth at 0.17 and 12.06 μM, respectively. The IC50–72 h for *R. subcapitata* exposed to Pb was 0.78 μM, almost 2-fold higher than reported by De Schamphelaere et al. (2014) also for *R. subcapitata* (0.40 μM). In Cd-exposure, the IC50–72 h of the present study (0.04 μM) was ≈ 10-fold lower than those reported in the literature, *e.g.* IC50–72 h of 0.5 μM of Cd (II) (Machado et al., 2015) for *R. subcapitata*, similarly to Monteiro et al. (2011), that reported 0.51 μM of Cd to *Scenedesmus obliquos*. Based on IC-72 h values, we observed higher toxicity of Cd compared to Pb, similarly to the results to *Chlorella sorokiniana* reported by Carfagna et al. (2013). Our IC50–72 h value was lower probably due to the higher percentage of free Cd in the test solutions (98.9%), compared to Machado et al. (2015) who reported about 45% of Cd in its free form. This may have contributed to the higher toxicity of Cd compared to Pb, in our study, since only about 70% of Pb was in its free form.

4.2. PAM-parameters

The loss of efficiency in OEC (increased values of F_0/F_V) during Pb-exposure can be explained due to displacing calcium, manganese (Mn) and chloride ions by Pb into the OEC (Sharma and Dubey, 2005), impairing the water photo-oxidation process. Cadmium may also substitute Mn in the OEC chemical reactions, probably causing a depletion of Φ_M due to damage in electron transfer mechanisms (Mallick and Mohn, 2003), which result in changes of OEC efficiency (Fig. 2). In accordance with that, other studies related the presence of Cd, Pb, silver, uranium and zinc to alterations in the flow of electrons at the water splitting site, which affects the efficiency of the OEC (Herlory et al., 2013; Peña-Vásquez et al., 2010). Thereby, we considered this parameter as a great tool to evaluate the extension of metal toxicity in algae photosynthetic apparatus.

Regarding Φ_M values, we believe that Cd modified the PSII activity and disassembled the PSII proteins of *R. subcapitata*, similarly to the mechanisms found in pea and bean plants exposed to Cd concentrations (Miao et al., 2005; Sharma and Dubey, 2005). In addition, Pb has the

Table 3
Biochemical parameters (pg cell^{-1}) of *Raphidocelis subcapitata* exposed to Cd and Pb. Lipid classes are HC (aliphatic hydrocarbons), TAG (triglycerides), FFA (free fatty acids), ALC (aliphatic alcohol), ST (free sterol), AMPL (acetone-mobile polar lipids) and PL (phospholipids). The statistical comparison for each metal was performed independently. Rows with different letters represent significant difference ($p < 0.05$).

		Lipid classes (pg cell^{-1})							Total lipids (pg cell^{-1})	Total carbohydrate (pg cell^{-1})
		HC	TAG	FFA	ALC	ST	AMPL	PL		
Cadmium (μM)	Control	0.19 ± 0.01^{a}	0.07 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}	0.40 ± 0.01^{a}	0.82 ± 0.05^{a}	1.79 ± 0.07^{a}	2.12 ± 0.31^{a}
	0.02	0.27 ± 0.09^{a,b}	0.02 ± 0.00^{a}	0.08 ± 0.00^{a,b}	0.06 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.71 ± 0.02^{a,b}	0.95 ± 0.21^{a,b}	2.76 ± 0.15^{a,b}	3.33 ± 0.45^{a,b}
	0.04	0.16 ± 0.01^{a}	0.10 ± 0.01^{a,b}	0.09 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.10 ± 0.00^{a,b}	0.64 ± 0.06^{a,b}	1.24 ± 0.13^{a,b}	2.55 ± 0.24^{a,b}	1.86 ± 0.56^{a}
	0.08	1.03 ± 0.02^{a,b}	0.11 ± 0.02^{a,b}	0.18 ± 0.00^{b}	0.10 ± 0.00^{b}	0.52 ± 0.02^{b}	1.41 ± 0.14^{b}	3.19 ± 0.01^{a,b}	6.42 ± 0.62^{b}	4.61 ± 0.87^{b}
	0.17	1.87 ± 0.19^{b}	0.87 ± 0.27^{b}	0.43 ± 0.10^{b}	–	0.39 ± 0.09^{b}	2.49 ± 0.28^{b}	7.06 ± 1.37^{b}	12.97 ± 1.33^{b}	7.39 ± 1.08^{c}
Lead (μM)	1.20	0.67 ± 0.09^{a,b}	0.15 ± 0.02^{b}	0.15 ± 0.03^{b}	–	0.67 ± 0.05^{a}	0.95 ± 0.01^{b}	3.91 ± 0.43^{b}	5.33 ± 0.35^{b}	4.66 ± 0.35^{a}
	2.41	0.73 ± 0.14^{a,b}	0.10 ± 0.00^{a,b}	0.08 ± 0.01^{a,b}	0.03 ± 0.01^{a,b}	0.10 ± 0.04^{a}	0.95 ± 0.07^{b}	2.45 ± 0.46^{a,b}	4.74 ± 0.73^{b}	6.07 ± 2.94^{a}
	4.82	1.03 ± 0.10^{b}	0.11 ± 0.02^{a,b}	0.06 ± 0.02^{a,b}	0.03 ± 0.00^{a,b}	0.11 ± 0.02^{a}	0.62 ± 0.00^{a,b}	2.30 ± 0.30^{a,b}	4.99 ± 0.67^{b}	4.99 ± 1.29^{a}
	12.06	1.01 ± 0.12^{b}	0.17 ± 0.04^{b}	0.23 ± 0.00^{b}	0.12 ± 0.00^{b}	0.19 ± 0.01^{a}	0.53 ± 0.02^{a,b}	3.28 ± 0.08^{b}	5.89 ± 0.81^{b}	3.76 ± 0.88^{a}

ability to displace some metal ions in photosynthetic processes, such as Mg^{2+}, Fe^{2+} and Ca^{2+}, resulting in a reduction of primary productivity due to impairment of photosynthetic apparatus (Dao et al., 2017), causing a decline in the quantum yields. According to our data, we can infer that the electron transport between algae photosystems was impaired due to damage in the OEC and water oxidation process, leading to a possible cause of ϕ'_M reduction (Juneau et al., 2002; Mallick and Mohn, 2003).

Concerning the quenching parameters, they are useful endpoints to evaluate to what extent the energy trapped by the PSII reaction centers was used for electron transport or was dissipated by non-photochemical processes (Herlory et al., 2013). In our study, the high values of qP in Cd and Pb exposure indicate that although the algae had the capacity to invest in photochemistry, it did not happen, as demonstrated by the reduction in the ϕ_M. The reduction in ϕ'_M after 72 h during Cd and Pb exposure suggests the activation of non-photochemical processes by the algae, with a small increase ($p < 0.05$) in the qN value at 0.17 μM of Cd. The constant qN values for both metals and the small decrease in NPQ values at certain Pb concentrations suggest that photo-protection mechanisms were damaged, similarly to results found by Juneau et al. (2002) and Thomas et al. (2013) to Chlorophyceae.

According to Carfagna et al. (2013), the possible explanation for the effects of Cd on photosynthesis could be the damage of thylakoid membrane and reaction centers, resulting in inhibition of the PSII. Besides that, Cd can substitute Mg^{2+} in chlorophyll molecules, altering their conformation and possibly causing a loss of function, due to the lower stability of these altered molecules (Baumann et al., 2009). In addition, the energy of these unstable chlorophyll molecules, when excited, can be accidentally transferred to oxygen molecules, resulting in ROS production (Thomas et al., 2013), similar to what occurred in the algae exposed to Cd in the present study.

4.3. Chlorophyll *a* content and biochemical composition

Despite the trend of a reduction in Chl *a* content in some Chlorophyceae exposed to metals (Carfagna et al., 2013; Martínez-Ruiz and Martínez-Jerónimo, 2015), our study found the opposite effect. Even with the damage caused by metal exposure to PSII electron transport activity, shown by the lower values of ϕ'_M, the highest concentrations of Cd and Pb induced an increase in Chl *a* content by about 6.1 and 8.5 times, respectively. Based on our results, we believe that the increase in intracellular Chl *a* content may be a strategy to increase the photosynthetic efficiency even under metal-stress conditions. Higher Chl *a* production may allow the algae to increase CO_2 fixation and allocate carbon to sugars and carbohydrates (Chia et al., 2015). In fact, we observed that the increase in Chl *a* level was accompanied by an increase in the total carbohydrates and lipid contents (Table 3), especially in Cd-exposure.

Increased carbohydrate content under metal stress may serve as a detoxification mechanism due to interactions between the negative charges of these compounds and metal cations (Kaplan et al., 1987). In fact, carbohydrate levels increased at the highest Cd concentrations (0.08 and 0.17 μM), although there were no significant changes in Pb-exposure, possibly due to the lower percentage of Pb in its free form in culture media (Tables S3 and S4). Since carbohydrates and sugars serve as the raw material of important organic carbon for the production of other biochemical components, such as lipids, proteins and nucleic acids (Chia et al., 2015), an increase in carbohydrate synthesis during metal stress is sometimes accompanied by an increase in lipid production (Dao et al., 2017), similarly to our results.

It is known that under environmental stress conditions, lipids are accumulated as energy reserves, allowing algae to survive (Hu et al., 2008). The increase in intracellular lipids can enable the maintenance of the redox homeostasis, since these biomolecules act as electron sinks, helping to combat oxidative damage caused by ROS (Chia et al., 2015; Hu et al., 2008). Therefore, this corroborates our study, which

correlates the greater ROS production, with a greater accumulation of lipids in Cd-exposure. In addition, it also explains the decrease in ROS production at 0.17 μM of Cd, since an increase (19- fold) was observed in lipid content at this concentration probably in an attempt to reduce the oxidative damage.

4.4. ROS assays

In our study, Cd induced *R. subcapitata* cells to high oxidative stress with, surprisingly 1.4-fold more relative ROS at 0.08 than at 0.17 μM of Cd after 72 h of exposure. Under stress conditions, microalgae can activate enzymatic and non-enzymatic antioxidant mechanisms, *e.g.* in allelochemical (Hong et al., 2009) and metal (Li et al., 2006) exposures, which actually can explain the decrease in ROS from 48 h to 72 h on Pb-exposure. In general, the toxicity of metals is related to their strong reactivity, which ends up inhibiting enzymatic activities and favors the generation of oxidative damage (Carfagna et al., 2013). When electron transport is blocked, these surplus electrons are transported to molecular oxygen, generating ROS (Qian et al., 2009). Some consequences of ROS formation are oxidative DNA damage, lipid peroxidation, photosynthetic apparatus damage and cell death (Sharma et al., 2012b).

4.5. Lipidic classes

The results of the present study revealed differences in the alteration of lipid classes of *Raphidocelis subcapitata* in response to each metal. Among lipid classes, fatty acids (FA) are the major component of algal lipids, usually affected by some metals (Guschina and Harwood, 2006), as observed in the present study by the FFA increase especially at the highest concentrations of Cd and Pb. It is known that light intensity above saturation may alter FA contents in some microalgae (Sukenic et al., 1989; Wagenen et al., 2012). Under stress conditions, the limit of the saturating irradiance of algae, *i.e.* the light intensity where photosynthesis saturation (I_k) begins can be reduced (Candido and Lombardi, 2018) or extended (Camargo and Lombardi, 2017). Considering this, we believe that in our study, Cd and Pb toxicity may have led to a reduction in the algae I_k, resulting in increased FFA values, which is corroborated by the increase of this class content at the highest concentrations of both metals.

AMPL is a lipidic class that is related to pigments and may contain 35–50% of chlorophyll (Lombardi and Wangersky, 1991). Only Cd-exposure led to AMPL increase at the highest concentration, thus explaining the positive correlation between Chl *a* and AMPL observed in PCA analysis (Fig. S3). At 12.6 μM of Pb, ALC content was 6 times higher than the value obtained for control cells. At 0.08 μM of Cd, an increase of 5 times in ALC content was observed, whereas no ALC was detected at the highest Cd concentration. In addition, sterols (ST) was the second class that most increased at 0.08 and 0.17 μM of Cd, while in Pb-exposure the ST level did not differ from the control group. Besides that, an increase in PL synthesis was observed during both Cd and Pb exposure, similarly to Chia et al. (2015). The increase in PL and ST is probably related to a mechanism of protection of the algal cells, which increases the thickness or fluidity of its membrane to hinder the ingress of the metal ions in the cytoplasm, thereby reducing the impact of the toxicity and internalization of the metal (Rocha et al., 2016).

We also observed an increase in TAG levels, especially at Cd-exposure. This increase probably occurred due to a reduction in the lipase gene transcription, resulting in a lower degradation of this lipid class, as observed by Yang et al. (2013) in diatoms under nitrogen limitation. It is known that the production of TAGs is generally increased under metal stress or nutrient limitation (Chia et al., 2015; Hu et al., 2008) as a defense mechanism, optimizing a rapid adaptive membrane reorganization (Sharma et al., 2012a), protecting the cells and repairing damage caused by stressor agents. Wagenen et al. (2012) reported TAG accumulation in *Nannochloropsis salina* in response to oxidative stress. In fact, we observed a greater increase of TAG content (12.4-fold) at the highest Cd concentration (0.17 μM), where a reduced ROS production was also observed compared with the other Cd concentrations. Our results indicate a correlation between these two parameters in *R. subcapitata* at Cd-exposure, thus explaining the opposite correlation between ROS and TAG in PCA analysis (Fig. S3).

4.6. Comparison of the multiple endpoints

Cd-exposure induced greater ROS generation regarding the control group in 48 and 72 h. At 72 h, the highest concentration (0.17 μM) led to a lower ROS generation than observed in the other lower Cd concentrations, probably due to the greater increase in lipids, carbohydrates and Chl *a* contents. As we mentioned before, the higher production of energy reserve compounds in Cd-treated algal cells probably act as a defense mechanism (electron sinks) against oxidative damage. Based on ϕ_M values, this strategy was efficient to protect the photosynthetic apparatus at 0.02, 0.04 and 0.08 μM of Cd. Compared to the control group, ROS generation increased (175.5%) more than F_0/F_V (43.6%), ϕ_M (13.7%) and ϕ'_M (20.5%). Considering this, algal growth inhibition probably occurred mostly due to the ROS production and, to a lesser extent due to impairment of photosynthetic apparatus.

After 72 h of Pb-exposure, we noticed great negative effects on ϕ_M and F_0/F_V values at all tested concentrations despite no observed difference in ROS production, which enabled us to infer that the impact on photosynthetic apparatus was the main cause of growth inhibition to *R. subcapitata*. Regarding photosynthetic endpoints, F_0/F_V (62.2%) and ϕ_M (17.7%) were the most affected parameters with respect to control group. Therefore, based on these results, we can deduce that the loss of efficiency of OEC had a great influence on ϕ_M reduction and consequently contributed to limiting the algal growth. Pb led to a large production of Chl *a* at all concentrations tested, which indicates an attempt to mitigate the damage on photosynthetic apparatus. Among all the endpoints evaluated, after 72 h of Pb-exposure, the Chl *a* content (8.5×) was the most affected in comparison to the control group, followed by lipids (3.3×) and F_0/F_V (1.62×) at 12.06 μM. In addition, we also observed an increase in those parameters even at the lowest Pb concentration, showing that these endpoints are the best tools to assess Pb toxicity.

Among the biochemical and photosynthetic parameters assessed in the present study, the ROS, lipid composition and carbohydrates were the most important to better understand Cd toxicity mechanisms to *R. subcapitata*, and F_0/F_V and ϕ_M were the most helpful endpoints to elucidate Pb toxicity mechanisms. Once the targets and toxicity mechanisms of a contaminant are known, it becomes easier to invest in studies to mitigate the effects of these compounds and to protect aquatic ecosystems more effectively.

5. Conclusions

Our study demonstrated that metals inhibited *R. subcapitata* growth, with IC50–72 h occurring at 0.04 for Cd and 0.78 μM for Pb. We identified distinctly toxicity mechanisms for each metal tested, where ROS production was the most probable mode of action of Cd toxicity, and impairment of photosynthetic apparatus the major cause of growth inhibition in Pb-exposed algal cells. *Raphidocelis subcapitata* changed its metabolic pathways differently to deal with each metal: in Cd-exposure, the algae increased the synthesis of total lipids (mostly FFA, ST and TAG) and carbohydrates, probably as a defense mechanism to manage the ROS effects and the damage to photosynthetic apparatus, while in Pb-exposure the increase of total lipids (mostly FFA, ALC and HC) and Chl *a* content were important to reduce ROS generation. Lipid class determination was effective as biomarkers for Cd and Pb toxicity.

Acknowledgments

The authors are thankful to the São Paulo Research Foundation

(FAPESP, Brazil) (Process nos. 2014/14139-3 and 2016/00753-7) for the financial support; and to the National Council for Scientific and Technological Development - CNPq, Brazil (Process nos. 305229/2016-8, 309514/2017-7 and 141126/2017-5) and to the Coordination for the Improvement of Higher Education Personnel - CAPES, Brazil (Process nos. 1715276 and 1586596) for the scholarship granted. We would also like to thank Dr. Ana Teresa Lombardi for the permission to use the PHYTO-PAM Fluorometer Analyser, Larissa Luiza dos Reis for the help during the experimental tests and M.S. Daniela Correia de Melo for helping HS with adjustments in ROS measurements.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.11.087.

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